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Pig-heart myoglobin and its derivatives with nitric oxide

The crystallization of Mb from horse hearts[1] and from sperm whale[2] has been accomplished, but similar preparations from pig muscle have been obtained only as amorphous products of doubtful homogeneity[3,4]. The preparation of purified MetMb from pig hearts has been undertaken; although crystallization was again not achieved, the product, isolated in the ferric form, was electrophoretically homogeneous and of essentially the same iron content and extinction coefficient at 630 mμ as crystalline horse-heart MetMb.

KEILIN AND HARTREE[5] were able to differentiate between stable NOHb and the unstable complex of nitric oxide with MetHb, and the ability of this gas to combine with the ferric form of iron–porphyrin compounds in general has been reported by EHRENBURG AND SZCZEPKOWSKI[6]; the latter authors stated that most of these compounds are unstable, instancing NOMetHb and NOMetMb as showing gradual autoreduction. So far as the haem pigments of the pig are concerned, this paper confirms the pattern of instability of NOMetHb but reports that pig NOMetMb is relatively stable anaerobically but is extensively degraded in air.

The centrifugation of aqueous extracts of minced washed pig hearts (0.82 l water per kg tissue) resulted in clear solutions to which a slight excess of basic lead acetate was added in the manner of THEORELL[7], the resultant heavy precipitate being centrifuged off. To such clear dark red solutions at pH 6.8, solid $(NH_4)_2SO_4$ was added with shaking; the original Mb fraction collected between 25 % and 45 % (w/v) of the salt at 0° was superseded by that precipitating between 38 % and 46 % (w/v) $(NH_4)_2SO_4$ which yielded products of significantly higher iron content and extinction coefficient at 630 mμ as isolated in the ferric form. The dark brown precipitates which separated at the higher $(NH_4)_2SO_4$ concentration were filtered off, taken up in water and dialysed exhaustively against running tap water. Final dialysis overnight against distilled water was followed by freeze-drying of the non-diffusible residue, the yield of brown feathery powder arising from fractionation between 38% and 46% $(NH_4)_2SO_4$ being of the order of 0.5 g per kg pig hearts. The iron content attained in this manner was 0.32 %, in agreement with the value reported for crystalline horse-heart Mb[1], and, unlike Hb[8], it was soluble in 3 M phosphate buffer (pH 6.8). In solution in 0.2 M phosphate buffer (pH 6.0), its mmolar extinction coefficient at its 630-mμ peak was 3.5 on the basis of one atom of iron per molecule, as compared with a value of 3.7 for horse-heart MetMb[1].

All attempts to crystallize purified pig MetMb by dialysis of its aqueous solutions at pH 6.8 in Visking cellulose casing against satd. $(NH_4)_2SO_4$ solution and by its maintenance in a 36 % solution of $(NH_4)_2SO_4$ at sub-precipitation level at 0° for several days, in a manner similar to that successfully employed by LAWRIE[9] for horse Mb, were unsuccessful, resulting only in amorphous powders. LEWIS AND SCHWEIGERT[4] attributed their inability to crystallize pig Mb to their failure to separate accompanying proteins by fractionation with $(NH_4)_2SO_4$, in the absence of a basic lead acetate precipitation stage. These contaminants were, however, detectable by electrophoresis, and the examination of a solution of purified pig MetMb in phosphate buffer (pH 9.3, I 0.5) in an Antweiler electrophoresis apparatus through the courtesy

Abbreviations: NOHb, NOMb, NOMetHb and NOMetMb: nitrosylhaemoglobin, -myoglobin, -methaemglobin, and -metmyoglobin, respectively.

of Mr. H. M. PAISLEY of the National Chemical Laboratory, Teddington, indicated one sharp symmetrical peak, anodic in migration. The application of the Mb–Hb differentiation technique of WALTERS AND TAYLOR[10] to three samples of pig-heart MbO_2 and pig HbO_2 over a wide range of concentrations resulted in reasonably constant ratios of the initial absorbancies at 577 mμ to those at the same wavelength after conversion to the corresponding alkali haemochrome at values of 1.51 ± 0.04 and 2.44 ± 0.02 (standard error of mean) respectively; this is in keeping with the observation of COLPA-BOONSTRA AND MINNAERT[11] that only Mb is generally found in carefully washed horse-heart muscle preparations. The pigment precipitated between 38% and 46% $(NH_4)_2SO_4$ was also homogeneous on Sephadex G-75, the material eluted with water giving the anticipated ratio of Soret (408 mμ) to ultraviolet (280 mμ) absorbancies of 4.5 in 0.05 M Tris buffer (pH 8.6) (see ref. 12).

As was reported by KEILIN AND HARTREE[5] for a Hb of unstated species, pig MetHb reacted with nitric oxide under anaerobic conditions to form a red complex showing maximum absorption at 535 and 570 mμ. Displacement of excess nitric oxide with argon resulted in the rapid appearance of an absorption spectrum indistinguishable from that of pig NOHb with maxima at 543 and 573 mμ.

Similarly, the anaerobic treatment of horse-heart MetMb (Nutritional Biochemicals Corpn., Cleveland, U.S.A.) in 0.2 M phosphate buffer (pH 6.0) with nitric oxide yielded a red complex (NOMetMb) whose spectrum revealed two maxima at 536 and 570 mμ sharper than those of horse NOMb and at shorter wavelengths. On standing in a closed cell, the α- and β-peaks of the spectrum of horse NOMetMb became less sharp and by 2.5 h they had increased in wavelength, a change consistent with partial autoreduction to NOMb[6], the spectral maxima of which were located at 548 and 580 mμ. The displacement of excess nitric oxide by bubbling with argon after the preparation of horse NOMetMb resulted in a breakdown of the compound with reversion to a brown solution with a spectrum indistinguishable from that of

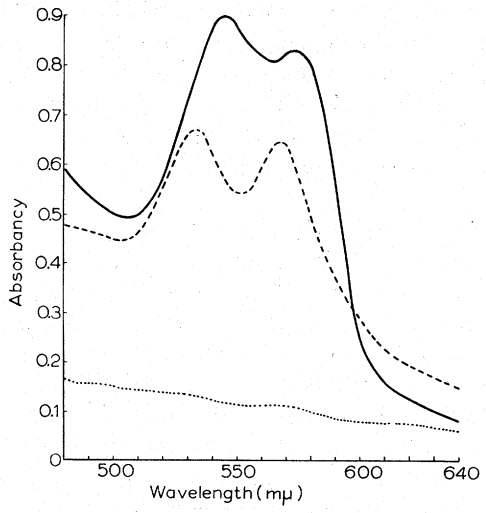

Fig. 1. Absorption spectra of pig NOMb (———), of pig NOMetMb (– – – –) and of the decomposition product of the latter in air (.....). Concn. of pigments 1.6 mg per ml 0.2 M phosphate buffer (pH 6.0). Light-path, 1 cm.

MetMb and which reacted again with nitric oxide under anaerobic conditions to yield a red complex with the spectral characteristics of NOMetMb.

Pig MetMb reacted in a similar manner with nitric oxide, the wavelengths of maximum absorption for the red NOMetMb complex being at 534 and 567 mμ (Fig. 1), as distinct from those at 545 and 574 mμ for pig NOMb. The pig NOMetMb complex survived the displacement of excess nitric oxide with argon, and detectable autoreduction did not take place at an appreciable rate; judging by the persistence of its spectral bands and by its instability on admission of air in comparison with stable pig NOMb, the NOMetMb complex was stable anaerobically at room temperature for at least 20 h. The admission of air, however, led to a rapid decomposition of the pig NOMetMb compound to yield a yellow-brown solution of absorption spectrum without pronounced peaks (Fig. 1). Light was not necessary for this decomposition and the end product did not react again with nitric oxide to yield a NOMetMb complex; treatment of the decomposition product gave no indication of the formation of reduced myoglobin and no haemochromogen formation with pyridine could be detected after reduction, although readily apparent with the original MetMb. In explaining the diminished stability of nitric oxide complexes with MetMb in comparison with those of cytochrome *c*, EHRENBURG AND SZCZEPKOWSKI[6] have stated that the reactions of myoglobin are compatible with a structure in which the haem is more exposed than in cytochrome *c*. If this hypothesis is applicable to the differences in behaviour between myoglobins of various species, it would imply that the degree of protection of the haem moieties of these myoglobins is variable, that of the pig being better protected than that of the horse. Nevertheless, pig NOMetMb is apparently susceptible to rapid and extensive degradation in air such that the haem moiety is no longer available; no suggestion can be advanced for the site of this attack or the source of the activation providing the enhanced sensitivity, although a similar instability to air has been observed with the nicotinic acid haemochromogen of pig myoglobin.

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British Food Manufacturing Industries Research Association, Leatherhead (Great Britain)

C. L. WALTERS
A. MCM. TAYLOR

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